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The rapid isolation of mutants of some Gram-positive bacteria

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1. INTRODUCTION

Mutant analysis facilitates, in several ways, the study of cellular processes at the molecular level. With the proper series of mutants, for example, it is possible to define the enzymatic steps in a biochemical pathway. With a multiply-marked strain it is possible to map the structural gene for a protein of particular interest. Knowledge of the location of a marker simplifies the task of preparing strains containing different combinations of mutations which can be used to analyse the regulation of particular biochemical pathways.

A number of problems have been encountered when attempts have been made to isolate, for the first time, a stock of mutants of a hitherto neglected organism. First, well-tried methods for obtaining mutants of several bacterial species are lacking. Second, since two organisms rarely will respond in precisely the same way to a mutagenic treatment, or a mutant screening programme, it often happens that a published procedure has to be modified to yield optimal results. Finally, provision must be made for segregation of pure mutant cells before cloning so as not to isolate strains all bearing exactly the same mutation. Thus isolation of independent mutants requires independent mutagenic treatments. In this communication we described a method for isolating at high frequency independent mutants of a number of Gram-positive bacteria. The method was originally developed for use with an *Arthrobacter* sp. and appears to work best with this and other coryneform bacteria.

2. MATERIALS AND METHODS

All the bacteria used were from the culture collections maintained at the University of Warwick or the Centre for Applied Microbiological Research. *Arthrobacter* P1 and coryneform D7F have been described elsewhere [1,2].

The complex media used were TSBA (for *Bacillus* sp.) and Oxoid nutrient agar (all other strains). TSBA contained, per litre, 17 g Oxoid tryptone, 3 g Oxoid soya peptone, 5 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose and 15 g agar; the pH was adjusted to 7.3 with 2 N HCl prior to sterilization. The minimal medium used was that of Spizizen [3] except for *Arthrobacter,* when the medium of Levering et al. [1] was used. Minimal media were supplemented with amino acids as required. Nutritional pool plates were prepared as described by Clowes and Hayes [4].

For mutagenesis using UV light, cells were grown in complex media and used while still in the logarithmic phase of growth. Details of the irradiation procedure are given in the text.

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3. RESULTS

Our original observations were made on a yellow pigmented, facultative methylotroph, *Arthrobacter* P1 (Levering et al., 1981). Portions (0.1 ml; approx. 10^7 cells) were spread on nutrient agar plates and irradiated with UV light for varying periods of time (20–90 s) until there were 10–1 000 survivors per plate. Survivors from this irradiation were tested for their ability to grow on minimal medium, and all potential auxotrophs were retested both on minimal and complex medium. In six separate experiments the percentages of auxotrophs isolated in this way were 3.0%, 4.0%, 4.3%, 6.7%, 2.2% and 2.4%. For most mutagens, the number of viable mutants in the population will increase with irradiation dose up to a certain level, and then fall as killing overtakes the induction of new mutants. However, at the sampling times used with this method, there was no significant difference in the number of auxotrophs obtained. Attempts were made to identify the metabolic lesion in all the auxotrophs by picking them onto nutritional pool plates. Approx. 40% of the mutants failed to grow on any pool plate indicating multiple nutritional requirements. The remainder were identified as having lesions in one of the following biosynthetic pathways: leucine, lysine (2 isolates), tryptophan, arginine, glycine, serine, valine, proline, adenine (3 isolates), nicotinic acid, biotin, histidine, isoleucine and threonine. Two other mutants were also identified, one probably with a defect in methylamine transport and the other with a defect in pigment biosynthesis.

Because of the ease with which mutants of *Arthrobacter* P1 could be isolated, we mutagenized a variety of bacteria in a similar way and screened survivors for auxotrophs. No auxotrophs were found for *Escherichia coli* strain C (0/550), *Aeromonas hydrophila* strain HY (0/1150), *Pseudomonas aeruginosa* strain PAT (0/728), *Pseudomonas oxalaticus* (0/950) or *Bacillus subtilis* strains UW1 and BD224 (0/522 and 0/1080). However auxotrophs were found with coryneform D7F (16/141). Approx. 38% of the D7F auxotrophs had complex requirements and the remainder had single requirements which could be identified by use of nutritional pool plates.

Auxotrophs were also found with the thermophilic *Bacillus licheniformis* strain, LO2. In the first experiment with this strain, trimethoprin (Tp)-resistant mutants were isolated. On media containing 15 μg/ml Tp they constituted 6.7% and 3.3% of survivors (0.003% and 0.002% survival, respectively) and on media containing 25 μg/ml Tp they constituted 3.8% and 1.0% of survivors. In 3 separate experiments the proportion of Thy^- auxotrophs among the Tp^R mutants ranged from 12–24%. For the isolation of other auxotrophs, strain LO2 was irradiated on plates to 0.003% survival. The frequency of auxotrophs among the survivors ranged from 0.7–1.3% and the frequency of identifiable auxotrophs from 0.14% to 0.2% of survivors. Mutants have been isolated in this way which have defects in the biosynthesis of histidine, leucine, isoleucine/valine, methionine, cysteine, arginine, lysine, guanine, and polyglutamyl polypeptide, and double and triple auxotrophs have been constructed. All the auxotrophic markers tested were stable having reversion frequencies ranging from $8.0 \cdot 10^{-7}$ to less than $1.5 \cdot 10^{-10}$.

Under most cultural conditions, bacterial cells rarely contain a single genome. Thus after mutagenesis it is essential to grow the cells for a few generations to allow segregation of mutant loci. This may be unnecessary if mutagenesis is associated with appreciable killing since any viable but mutant genome is unlikely to find itself in the same bacterium as a viable but non-mutant genome. With the mutagenesis method described above there is no need to allow segregation of mutant loci. Furthermore, appreciable killing occurs and so inactivation of non-mutant genomes is highly probable. However, if killing of non-mutant genomes is the explanation for the success of the method then it should work with all bacteria. The failure to obtain mutants of a number of bacteria suggests that there is an alternative explanation. One possibility is that *Arthrobacter* P1, coryneform D7F and *B. licheniformis* normally have only one or two genomic copies whereas the other strains tested have three or four. If this were the case then lowering of the genomic copy number might facilitate mutagenesis. Consequently, *E. coli* and *A. hydrophila* were grown in nutrient broth and in chemostats at a dilution rate of 0.05/h and their

susceptibility to mutagenesis tested as described above. No auxotrophs were isolated regardless of the growth conditions.

4. DISCUSSION

The method of direct mutant isolation described in this communication has a number of advantages over conventional methods. First, it is extremely rapid and simple and requires no calibration of the UV source. Second, a range of different auxotrophs can be isolated from a single experiment. Third, because there are no detectable auxotrophs prior to irradiation, different isolates with the same phenotype obtained from the same plate can be assumed to be independent mutants. This is a particularly atttractive feature if the aim of the mutants is to facilitate analysis of a biochemical pathway and is the major advantage over conventional methods of mutant isolation (see [5] for discussion). The biggest disadvantage with the method is that undoubtedly most of the auxotrophs isolated will carry multiple mutations. This is clear from the fact that a high proportion of them could not be characterized nutritionally. Even with the auxotrophs which could be identified there is still a high probability that they carry other "silent" mutations. Whether or not these prove troublesome would depend on the circumstances for which the mutants are to be used.

The reason why the method described here only works with certain organisms is not known. However, since the aim of the method was to obtain a stock of mutants and not to study mutagenesis itself, this hardly matters. Because of the simplicity of the method we recommend that it be tried first, before the conventional methods, when developing the genetics of a hitherto neglected organism.

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